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# Expression and Distribution of Osteopontin in Human Tissues: Widespread Association with Luminal Epithelial Surfaces

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Osteopontin, a glycoprotein with a glycine-arginine-glycine-aspartate-serine (GRGDS) cell-binding domain, has been described in bone and is also known to be expressed in other organs, particularly kidney. The goal of the present work was to define the distribution of osteopontin synthesis and deposition in a wide variety of normal adult human tissues using a multifaceted approach that included immunohistochemistry, in situ hybridization, and Northern analysis. Immunohistochemical studies have revealed the unexpected finding that osteopontin is deposited as a prominent layer at the luminal surfaces of specific populations of epithelial cells of the gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lung, breast, salivary glands, and sweat glands. Northern analyses identified gallbladder as a major site of osteopontin gene transcription comparable in magnitude with that of kidney, and immunoblotting identified osteopontin in bile. In situ hybridization localized osteopontin gene transcripts predominantly to the epithelium of a variety of organs as well as to ganglion cells of bowel wall. Osteopontin of epithelial cell origin, like bone-derived osteopontin, promoted GRGDS-dependent cell spreading in attachment assays. We postulate that osteopontin secreted by epithelium binds integrins on luminal surfaces. Collectively, these findings suggest an important role for osteopontin on many luminal epithelial surfaces communicating with the external environment.

## INTRODUCTION

Osteopontin (secreted phosphoprotein 1, 2 ar) is a secreted adhesive glycoprotein with a GRGDS cell-binding domain (Oldberg *et al.*, 1986; Craig *et al.*, 1989; Kiefer *et al.*, 1989; Wrana *et al.*, 1989). This motif is present in fibronectin, vitronectin, and a variety of other extracellular proteins that bind members of the integrin family of cell surface receptors (Ruoslahti, 1991; Hynes, 1992). Consistent with the presence of a GRGDS cell-binding sequence, substratum-bound osteopontin from bone promotes attachment and spreading of cells in vitro, and cell attachment is blocked by soluble GRGDS peptides (Oldberg *et al.*, 1986; Somerman, *et al.*, 1989). Moreover, a monoclonal antibody to the integrin $\alpha_v\beta_3$ has been reported to block osteopontin-stimulated changes in osteoclast cytosolic calcium, suggesting that osteopontin is a ligand for integrin $\alpha_v\beta_3$ (Miyauchi *et al.*, 1991).

The prominence of osteopontin in bone matrix and the regulation of its expression by osteotropic hormones have focused attention on the role of this protein in bone development and remodeling (Prince and Butler, 1987; Yoon *et al.*, 1987; Kubota *et al.*, 1989; Noda *et al.*, 1990; Reinholt *et al.*, 1990; Chen *et al.*, 1991; Moore *et al.*, 1991). However, several laboratories have shown that osteopontin mRNA is expressed by a number of tissues, including kidney, ovary, uterus, and others (Yoon *et al.*, 1987; Nomura *et al.*, 1988; Denhardt *et al.*, 1989; Young *et al.*, 1990; Craig and Denhardt, 1991). Thus, prior studies have suggested that osteopontin is expressed in tissues other than bone; however, the specific cellular sites of osteopontin synthesis and deposition in most of these tissues have not been investigated,

and other tissues have never been examined for osteopontin expression. Therefore, to provide a more comprehensive picture of the normal tissue and cell distribution of osteopontin mRNA and protein, we prepared RNA and cDNA probes and affinity-purified antibodies and used these reagents to perform in situ hybridization, Northern analyses, and immunohistochemistry on a variety of normal adult human tissues. Our studies have revealed a previously unrecognized and unifying pattern of osteopontin expression and localization in normal adults, i.e., this adhesive protein is present on luminal surfaces of many epithelial cells communicating with the external environment.

## MATERIALS AND METHODS

### *Osteopontin Purification and Affinity-Purified Antibodies*

Osteopontin was purified to homogeneity from human milk as described previously (Senger *et al.*, 1989b). As determined by sodium dodecyl sulfate (SDS) polyacrylamide gels, these osteopontin preparations contained a single protein, and N-terminal sequence analyses revealed a single sequence identical to that of osteopontin (Senger *et al.*, 1989a,b). For additional assurance of protein purity, osteopontin for immunization of rabbits was subjected to electrophoresis in SDS polyacrylamide gels, the gels were stained, and the osteopontin bands were excised. Rabbits were immunized with pulverized polyacrylamide slices containing 60 μg osteopontin together with complete Freund's adjuvant and boosted twice with one-half the amount of the same antigen mixed with incomplete adjuvant at 3-w intervals. Antisera obtained stained osteopontin antigen on immunoblots. To eliminate other antibodies that might be present in whole rabbit serum, we affinity-purified osteopontin antibody. For affinity-purification of antibody, an osteopontin-Sepharose column was prepared by coupling 300 μg purified osteopontin to 3.5 ml cyanogen bromide-activated Sepharose (Pharmacia, Piscataway, NJ). Fifteen milliliters of antiserum was passed over the column, and after washing, bound antibody was eluted with 15 ml 0.1 M glycine, pH 2.5, and immediately neutralized with 2 ml of 1 M tris(hydroxymethyl)aminomethane, pH 8.0. Affinity-purified antibody preparations obtained were of concentrations typically 0.3–0.6 mg/ml. To assure for specific-staining, only affinity-purified antibody was used in immunohistochemistry (see below).

### *Tissue Selection*

Immunohistochemical staining was performed on archival formalin-fixed paraffin-embedded material. Blocks containing normal tissue without microscopic abnormalities were chosen for these studies. Fresh tissues for Northern blots and in situ hybridization were selected from grossly normal areas of resected surgical specimens.

### *Immunohistochemical Staining*

All staining was performed on the Histomatic Slide Stainer, Code-On Series (Fisher Scientific, Pittsburgh, PA). Staining was accomplished in a two-step procedure using affinity-purified osteopontin antibody typically at 1:50 dilution, followed by peroxidase-labeled anti-rabbit IgG antibodies. Final color development was achieved with 3-amino-9-ethyl carbazole. Slides were counterstained with hematoxalin. Negative controls were performed substituting rabbit IgG at a concentration equal to that of the affinity-purified osteopontin antibody.

### *cDNA Cloning and Probe Preparation*

A human mammary gland cDNA library in λgt11(HL1037b) was obtained from Clontech Laboratories (Palo Alto, CA). Plaques were obtained by plating the library on *Escherichia coli* strain Y1090 and screened with affinity-purified osteopontin antibody. A total of $1.8 \times 10^5$ phage plaques were screened, and one positive clone was obtained. The positive plaque was recovered from agar and replated for secondary screening. DNA was prepared from the positive clone by polyethylene glycol precipitation of phage lysate followed by phenol/chloroform extraction and ethanol precipitation. The cloned insert was recovered by polymerase chain reaction (PCR) amplification with λgt11-specific primers (New England Biolabs, Beverly, MA). The cloned insert (~1.5 kilobase [kb] in size) was then subjected to restriction mapping that confirmed that it represented osteopontin cDNA (Kiefer *et al.*, 1989). Further proof of the identity of the λgt11 clone was obtained by subcloning its 330-base pair (bp) portion located between *Eco*RI and *Hin*dIII restriction sites. This DNA fragment was inserted into an M13 vector and sequenced using the chain termination method (Sequenase kit, United States Biochemical, Cleveland, OH). The DNA sequence is identical to the previously reported osteopontin cDNA sequence (Kiefer *et al.*, 1989). The entire 1.5-kb PCR product was used as a probe in the Northern blotting experiments (see below).

### *Northern Analysis*

RNA isolation and blotting were performed as described previously (Berse *et al.*, 1992). Briefly, frozen tissues were ground in a mortar in presence of liquid nitrogen and then lysed with guanidinium isothiocyanate. RNA was pelleted through a CsCl gradient, precipitated with ethanol, and stored as ethanol precipitates at −80°C. For Northern analysis, RNA samples that had been normalized for ribosomal RNA content (20 μg) were size-fractionated on agarose/formaldehyde gels and transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Hybridization was carried out overnight at 42°C in 50% formamide, 5× SSPE, 2.5× Denhardt's solution, 0.1% SDS, and 10% dextran sulphate (Sambrook *et al.*, 1989). DNA probes were labeled with [$\alpha$-$^{32}$P]dCTP (New England Nuclear, Boston, MA) to a specific activity of $1–2 \times 10^9$ cpm/μg DNA using the oligolabeling kit (Pharmacia). Final washes were in 2× SSC, 0.1% SDS at 65°C. The blots were exposed to Kodak (Rochester, NY) XAR-2 film with an intensifying screen at −70°C, typically for 1–4 d. To control for total mRNA content and lack of degradation, the blots were stripped and subsequently rehybridized with a human β-actin probe (Clontech Laboratories).

### *In Situ Hybridization*

To construct a template for probe synthesis, the 330-bp *Eco*RI-*Hin*dIII fragment of the osteopontin cDNA (corresponding to the 3′ end of the coding region and extending 200 bp into the 3′ untranslated region) was inserted into plasmid pGEM-3Zf(+) (Promega, Madison, WI). To obtain the antisense RNA probe, the pGEM construct with osteopontin insert was linearized with *Eco*RI and transcribed in vitro from the SP6 polymerase promoter; alternatively, the same construct was linearized with *Hin*dIII and transcribed from the T7 promoter to yield the sense (control) probe. Transcription reactions were carried out using a Riboprobe Gemini II kit (Promega) in the presence of [$\alpha$-$^{35}$S]UTP (Dupont New England Nuclear Products, Boston, MA). The probes were purified by gel electrophoresis, and in situ hybridization on tissues processed through paraffin was performed as described previously (Berse *et al.*, 1992). In addition, some in situ hybridization experiments were performed on frozen sections. Tissue was fixed for 4 h at 4°C in 4% paraformaldehyde (prepared in phosphate-buffered saline [PBS]) and then transferred to 30% sucrose in PBS at 4°C overnight. Tissue was then embedded in O.C.T. compound (Miles Laboratories, Elkhardt, IN), and 5-μm frozen sections were cut on a cryostat. In situ hybridization on frozen sections was performed as on paraffin sections but deparafinization steps were omitted.

### *Immunoblotting of Bile Proteins*

Barium citrate adsorption of osteopontin from bile was performed as described previously for adsorption of osteopontin from blood plasma

**Table 1.** Organ, tissue, and cell distribution of osteopontin as determined by immunohistochemistry (IH), in situ hybridization (ISH), and Northern analysis

| Organ | Northern analysis | Histologic localization | IH | ISH |
|---|---|---|---|---|
| Breast, lactating | | Lactating epithelium, luminal contents | + | |
| Breast, nonlactating | + | Duct epithelium | + | |
| Bone | | Cement line, edges of bone trabeculae | + | |
| Stomach | + | Surface mucous epithelium | + | |
| Small and large intestine | + | Epithelium | + | − |
| Colonic wall exclusive of mucosa | + | Ganglion cells | + | + |
| Salivary glands | | Duct epithelium, mucinous epithelium | + | |
| Liver | + | Bile duct epithelium | + | |
| Gallbladder | + | Epithelium | + | + |
| Pancreas | | Duct epithelium | + | + |
| Kidney | + | Distal tubular epithelium | + | + |
| Bladder | | Epithelium | + | |
| Prostate | | Gland epithelium | + | |
| Testis | | Epithelium, mediastinum testis | + | |
| Endocervix | + | Epithelium | + | |
| Endometrium | + | Secretory endometrium | + | + |
| Fallopian tube | | Epithelium | + | + |
| Ovary | | Mesothelium | + | |
| Placenta | | Amnion lining cells, stromal cells in villi | + | |
| Lung | + | Bronchial epithelium, mucous gland epithelium | + | |
| Skin | | Sweat ducts, sweat glands | + | |
| Mesothelium | | Mesothelial lining cells | + | |
| Lymph node | | | − | |
| Spleen | | | − | |
| Skeletal muscle | | | − | |
| Connective tissue | | | − | − |

+, presence of osteopontin mRNA (by Northern analysis or ISH) or presence of osteopontin (by IH). Blank spaces indicate that tests were not performed.

and serum (Senger *et al.*, 1988) and adsorption of osteopontin from breast milk (Senger *et al.*, 1989b). The barium-citrate adsorbed proteins from 40 ml of bile were eluted with 4 ml of 0.2 M of sodium citrate, pH 6.8, and 80-μl aliquots of eluate were subjected to SDS polyacrylamide gel electrophoresis (Laemmli, 1970), followed by a transfer to nitrocellulose as described previously (Senger *et al.*, 1988). Samples were heated and reduced before electrophoresis. Blots were stained with osteopontin antiserum or control rabbit serum followed by [$^{125}$I] protein A (Dupont New England Nuclear Products) and visualized with autoradiography (see Northern blotting, above).

### *Cell Lines and Cell Attachment Assays*

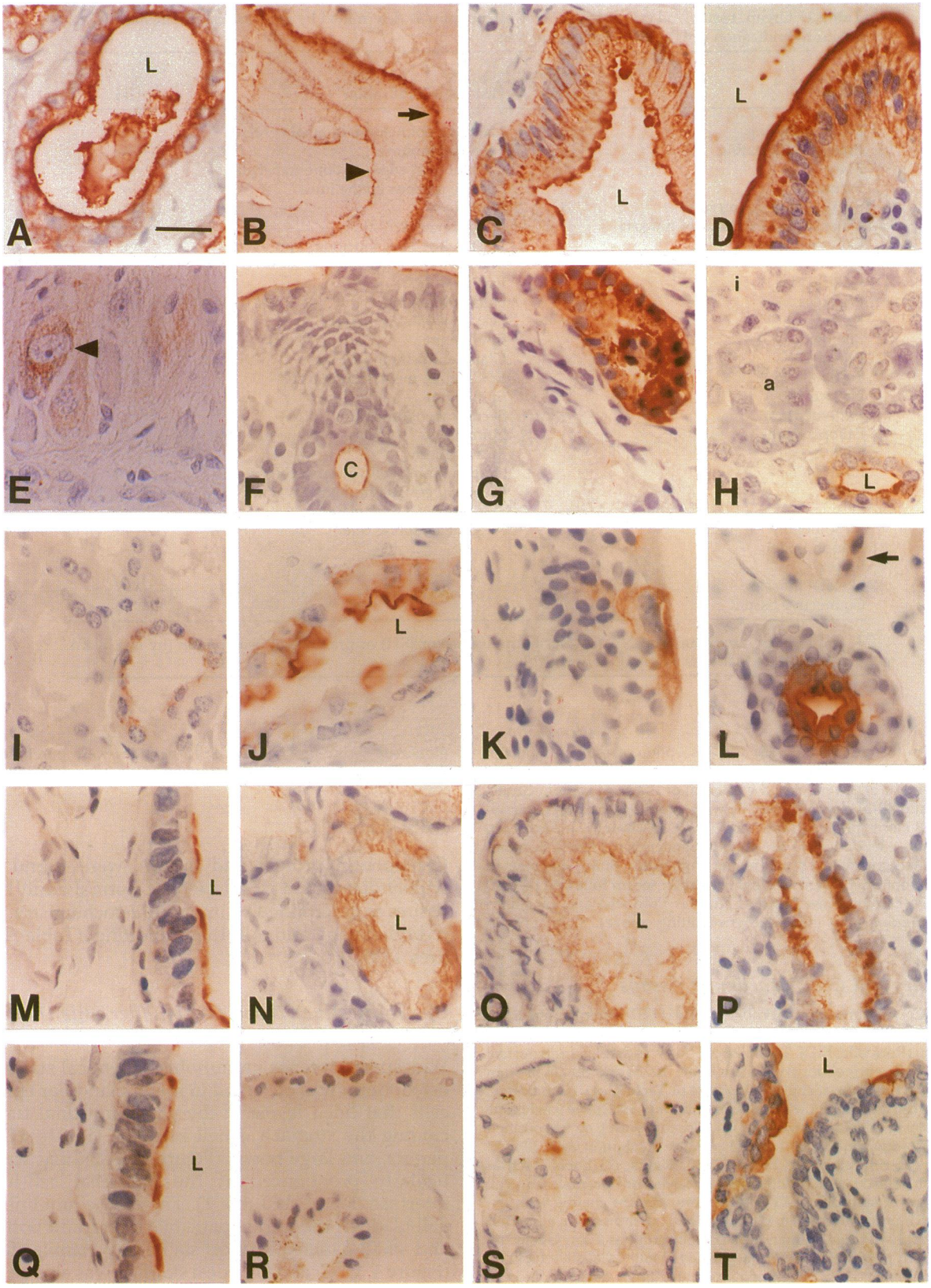
CCD-18Co human colon fibroblasts and HISM human intestinal smooth muscle cells were obtained from the American Type Culture Collection (Rockville, MD). The K-16 cell line was derived from normal adult rat liver epithelium (Weinstein *et al.*, 1975) and obtained from Dr. R. O. Hynes, Massachusetts Institute of Technology (Cambridge, MA). For cell attachment assays (Ruoslahti *et al.*, 1982), we used tissue culture plastic (Costar, Cambridge, MA) coated with 6 μg/ml purified human osteopontin (37°C, 1 h) followed by incubation with 20 mg/ml bovine serum albumin (BSA, A7030; Sigma, St. Louis, MO) at 37°C, 1 h. Plastic coated with BSA alone did not promote any cell attachment or spreading. We found more efficient coating with osteopontin if performed in 0.1 M glycine, pH 3.0, rather than at neutral pH, probably because human milk osteopontin has a low isoelectric point (about pH 3.0–3.5; our unpublished results). Cells were plated in serum-free medium; attachment and spreading was monitored microscopically for up to 24 h. GRGDSP and glycine-arginine-glycine-glutamate-serine-proline (GRGESP) peptides were purchased from Telios Pharmaceuticals (San Diego, CA).

## RESULTS

Osteopontin protein and mRNA were found to be widely distributed in a variety of normal adult human tissues as determined by immunohistochemistry, in situ hybridization, and Northern blotting (Table 1, Figures 1–4).

### *Gastrointestinal Tract, Gallbladder, Bile Ducts, Pancreas, and Salivary Glands*

Widespread intense immunohistochemical staining for osteopontin was observed in many glandular epithelial cells that lined levels of the gastrointestinal tract, including the stomach, small intestine (Figure 1D), appendix, and large bowel (Figure 1F); staining was particularly pronounced at the epithelial cell-luminal interface (Figure 1, Table 1). The epithelium lining the gall bladder, the intrahepatic bile ducts, and both large and small pancreatic ducts stained in a similar fashion (Figure 1, C, G, and H). Focal staining was also observed in the duct epithelium of the salivary glands and in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mucinous acinar cells of the salivary glands. Of interest, ganglion cells of the intermuscular neural plexuses of the gastrointestinal tract also stained intensely for osteopontin (Figure 1E).

These immunohistochemical findings were supported further by Northern blotting and in situ hybridization. A 1.5-kb osteopontin transcript was found by Northern analyses in gallbladder, small intestine, colonic mucosa, colonic wall, stomach, and liver (Figure 2, Table 1). Relative to other tissues, gallbladder contained particularly high levels of osteopontin mRNA. Northern analyses detected relatively low levels of osteopontin mRNA in scrapings of colonic mucosa, though higher levels were found in colonic wall. In situ hybridization demonstrated osteopontin mRNA in gallbladder epithelium (Figure 3, a–d) and in the epithelium of pancreatic ducts (Figure 4, c and d). In the few cases examined, osteopontin transcript was not detected in intestinal epithelium with in situ hybridization; however, the intermuscular ganglion cells were strongly positive (Figure 3, g and h). In view of these findings, it is likely that the osteopontin mRNA detected in colonic wall by Northern analysis represented osteopontin mRNA present in bowel wall ganglion cells rather than muscle cells, which contained no detectable osteopontin (by immunohistochemistry) or osteopontin mRNA (by in situ hybridization).

In accord with these immunohistochemical and mRNA analyses, immunoblotting revealed the presence of osteopontin in bile (Figure 5). Whereas osteopontin isolated from human milk exhibited an electrophoretic mobility of approximately $M_r$ 75 000 (Senger *et al.*, 1989b), bile proteins contained not only that species but also lower and higher molecular weight forms. The former may represent proteolytic breakdown products, whereas the latter may represent polymers formed through covalent cross-linking of osteopontin to itself or to other bile proteins (see DISCUSSION). Taken together, these data indicate that osteopontin mRNA is transcribed and that osteopontin protein is synthesized in the gallbladder epithelium and subsequently secreted into the bile.

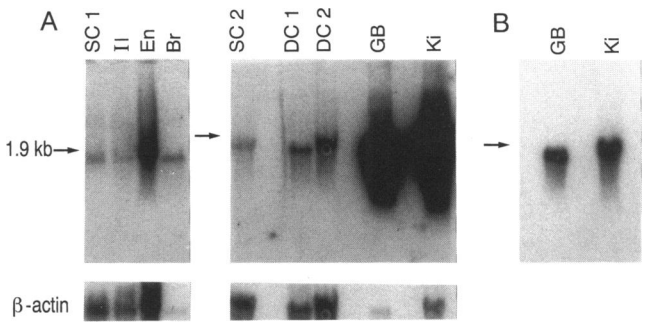



**Figure 2.** Distribution of osteopontin mRNA in several normal adult human tissues as determined by Northern blotting, using probes for osteopontin (upper) and β-actin (lower). The position of 1.9 kb β-actin mRNA is indicated in the upper panels with arrows. (A) SC1 and SC2 superficial colon (mucosa) samples 1 and 2. Il, ileum; En, endometrial epithelium; Br, breast; DC1 and DC2, deep colon (wall with muscle layers) samples 1 and 2; GB, gallbladder; Ki, kidney. Exposure time: 66 h. (B) Same as A, but short exposure (2.5 h). Distortion due to sample composition appears to be responsible for the slight variations in mRNA mobility. Relative quantities of osteopontin transcript, similar to that detected in superficial colon mucosa (SC), were also found in stomach, liver, and lung (not shown).

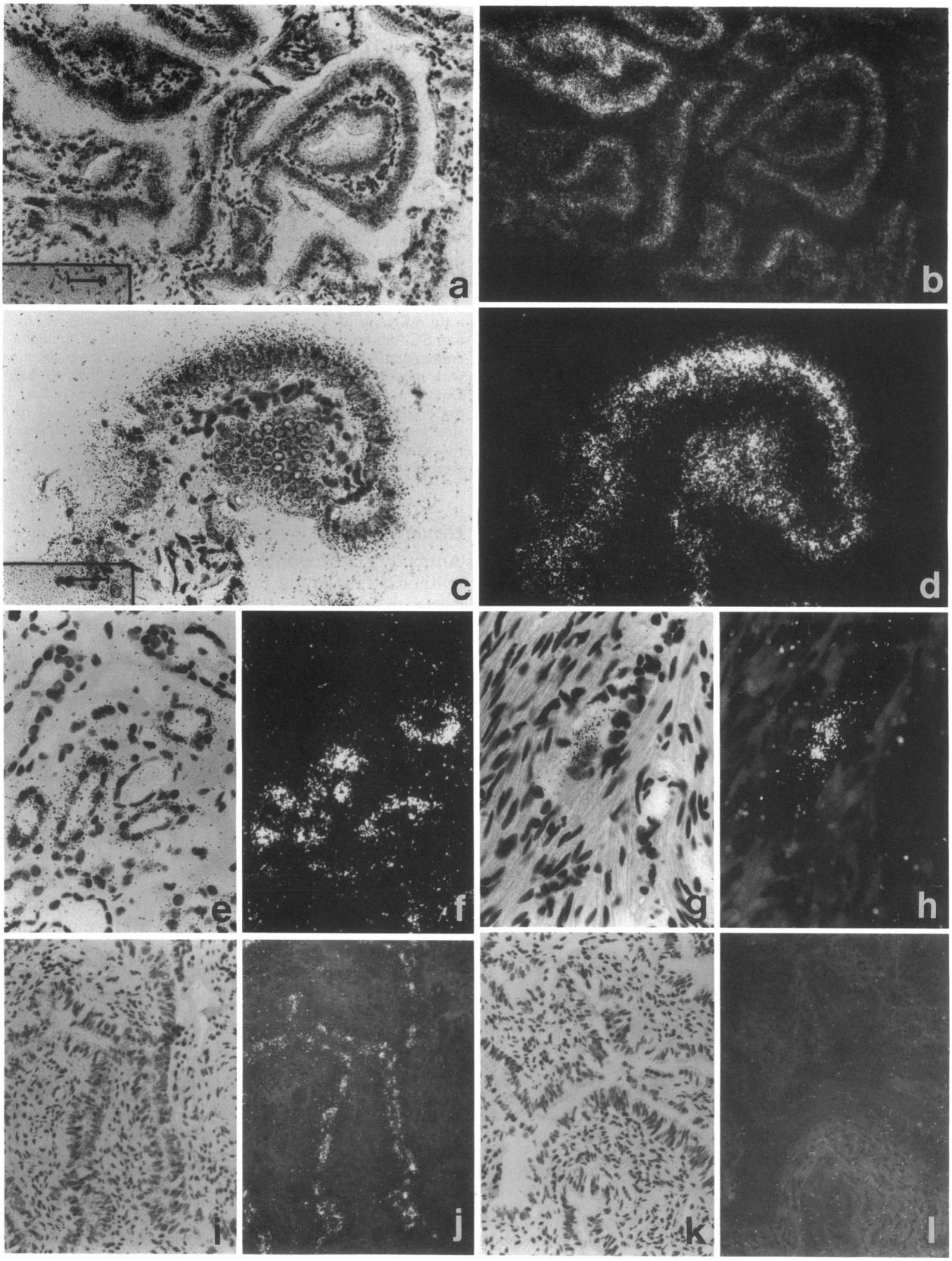
### *Urinary Tract*

Antibody to osteopontin stained the cytoplasm of many epithelial cells lining the distal tubules and collecting ducts of the kidney (Figure 1, I and J), as well as the transitional epithelium of the renal pelvis. In accord with these findings, in situ hybridization localized osteopontin mRNA predominantly to epithelial cells of the distal tubules (Figure 3, e and f), and Northern analysis of normal adult kidney revealed relatively high levels of 1.5 kb osteopontin mRNA (Figure 2). Antibody also stained the transitional epithelium of the bladder focally, limited in some cases to the most superficial epithelial cells (Figure 1K).

### *Male and Female Genital Tracts*

In the male genital tract, immunostaining for osteopontin was observed focally in epithelial cells of the prostate (Figure 1T) and in ductal epithelium of the mediastinum

**Figure 1.** Immunohistochemical staining of normal adult human tissues for osteopontin. Positive reactions are denoted by red-colored reaction product. L, lumen. In all cases, replacement of specific anti-osteopontin antibody with control rabbit IgG resulted in no staining. Magnifications are indicated by bar that equals 25 μm. (A) Glandular epithelium of lactating breast stains for osteopontin; staining is accentuated at the luminal surface and luminal contents also stain. (B) Bone (rib): edges of bone trabeculae (arrow) as well as cement lines (arrowhead) stain. (C) Gallbladder epithelium stains with supranuclear and luminal accentuation. (D) Small bowel epithelium stains with supranuclear and luminal accentuation. (E) Several ganglion cells within the myenteric plexus of small bowel muscle stain for osteopontin; the most intensely stained ganglion cell is indicated with an arrowhead. (F) Colonic epithelium stains with accentuation both at the luminal surface (top) and within a crypt lumen (c, bottom). (G) Intrahepatic bile duct epithelium stains, whereas hepatocytes (lower left) do not. (H) Pancreatic duct epithelium stains with luminal accentuation (lower right). Acini (a, center) and islets (i, upper left) do not stain. (I) Epithelial cells lining distal tubules of renal cortex stain (right); proximal tubules do not stain (left). (J) Collecting duct epithelium of renal medulla stains with luminal accentuation. (K) Superficial transitional epithelial cells lining the bladder stain. (L) Epithelium lining sweat ducts (bottom) of skin stain with luminal accentuation, whereas the sweat glands stain less intensely (arrow, top). (M and N) Lung: bronchial epithelium (M) stains focally with luminal accentuation and bronchial mucous glands (N) stain focally. (O) Endocervical glands stain. (P) Endometrial secretory glands associated with pregnancy stain. (Q) Fallopian tube epithelium stains with luminal accentuation. (R and S) Placenta: rare cells lining the amniotic cavity stain (R), as do occasional cells within the placental villi (S). (T) Prostate: glandular epithelium stains focally with luminal accentuation.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testes; as in many other epithelia, staining was accentuated at the luminal surface.

In the female genital tract, the mucinous epithelium of the endocervix stained strongly and diffusely with antibody to osteopontin, as did the hypersecretory endometrial gland epithelium associated with pregnancy (Figure 1, O and P). Secretory endometrium taken from nonpregnant subjects also stained focally and *in situ* hybridization demonstrated osteopontin transcript in the secretory gland epithelium (Figure 4, a and b). Northern blotting identified osteopontin mRNA in extracts of superficial endometrial and endocervical tissue (Figure 2), whereas at comparable autoradiographic exposures none was detected in uterine muscle. The epithelium lining the fallopian tubes reacted focally with anti-osteopontin antibody (Figure 1Q), giving an accentuated band of staining at the luminal surface and *in situ* hybridization localized osteopontin mRNA to luminal epithelial cells (Figure 3, i–l). In the ovary, antibody-staining was limited to surface mesothelial-like cells.

### *Osteopontin in Breast, Bone, Lung, Skin, and Other Tissues*

Luminal contents of lactating breast stained strongly for osteopontin as did the lining glandular epithelium; staining was accentuated at the luminal surface of epithelial cells (Figure 1A). In contrast, staining in sections of nonlactating breast was limited to occasional duct epithelial cells. Our antibody to milk osteopontin recognized osteopontin in bone, demonstrating prominent immunohistochemical staining of the cement lines and surfaces of mature bone trabeculae (Figure 1B). In the lung, antibody to osteopontin stained bronchial columnar epithelium focally, most prominently at the luminal surface (Figure 1M). Staining was also observed in epithelial cells of the bronchial mucous glands (Figure 1N). The presence of osteopontin mRNA in adult human lung was confirmed by Northern blotting. In the skin, strong immunostaining was found in sweat duct epithelium with an accentuated band of staining at the luminal surface; weaker staining was also observed in sweat gland epithelium (Figure 1L). Mesothelial cells lining the peritoneal surface stained focally with osteopontin antibody. Immunostaining in the placenta was found in isolated cells lining the amniotic cavity and in occasional cells within the stroma of placental villi (Figure 1, R and S).

Finally, a variety of tissues did not stain detectably with osteopontin antibody; these included lymph nodes, spleen, skeletal muscle, and connective tissue.

### *Cell Attachment and Spreading Directed by Epithelial Osteopontin*

Osteopontin isolated from bone has been shown previously to promote cell attachment and spreading (Oldberg *et al.*, 1986; Somerman *et al.*, 1989). To determine if osteopontin secreted by epithelium is also capable of promoting cell attachment and spreading, we purified osteopontin to homogeneity from human milk and used it in cell attachment assays. For these experiments, plastic wells were initially coated with osteopontin (6 μg/ml), and then, to eliminate nonspecific adherence of cells to plastic, wells were blocked with BSA (20 mg/ml). Thus, any cell attachment and spreading observed were directed by osteopontin and not by the underlying plastic substratum. As shown in Figure 6 (a–c), osteopontin purified from milk promoted attachment and spreading of human colon fibroblasts, rat epithelial cells derived from normal rat liver, and human intestinal smooth muscle cells. Cell attachment appeared to be dependent on the arginine-glycine-aspartate (RGD) domain of osteopontin because soluble GRGDSP peptide (300 μM) blocked this process completely, whereas control peptide GRGESP was without effect. No cell attachment occurred in wells coated with BSA alone (Figure 6d).

## DISCUSSION

In this study, we have demonstrated for the first time widespread presence of osteopontin on specific populations of luminal epithelial cells in the human gastrointestinal tract, gallbladder, bile ducts, pancreatic ducts, urinary and reproductive tracts, bronchi, lactating breast, sweat ducts and glands, and salivary ducts and glands. Moreover, we have demonstrated widespread expression of osteopontin mRNA by luminal epithelia and have determined that the gallbladder is a major site of osteopontin expression, comparable in magnitude with that of kidney. Prior studies with animal models identified osteopontin mRNA in several tissues and organs; however, in most cases, the cellular localization of the protein and the identity of the cells responsible for its synthesis were not established. Our findings of widespread accumulation of osteopontin on the surfaces

**Figure 3.** In situ hybridization on human tissue sections with osteopontin probes. Magnifications of a, b, and i–l are indicated by bar in panel a that equals 50 μm; magnifications of c–h are indicated by a bar in c that equals 25 μm. (a) Gallbladder, bright-field, osteopontin antisense (AS) probe. (b) Gallbladder, dark-field (AS). Note distinct epithelial labeling. (c) Gallbladder, bright-field, higher power (AS) (d) Gallbladder, dark-field, higher power (AS) (e) Kidney, medulla, bright-field (AS) (f) Kidney, medulla, dark-field (AS). Note labeling of selected distal tubular epithelium. (g) Muscular wall, colon, bright-field (AS) (h) Muscular wall, colon, dark-field (AS). Note labeling of ganglion cells. (i) Fallopian tube, bright-field (AS) (j) Fallopian tube, dark-field (AS). Note distinct epithelial labeling. (k) Fallopian tube, osteopontin sense probe (S), bright-field. (l) Fallopian tube (S), dark-field. Note low background with little or no epithelial labeling. Similarly low backgrounds and absence of any epithelial labeling patterns were seen with sense control probes hybridized to gallbladder, kidney, and colon (not shown).

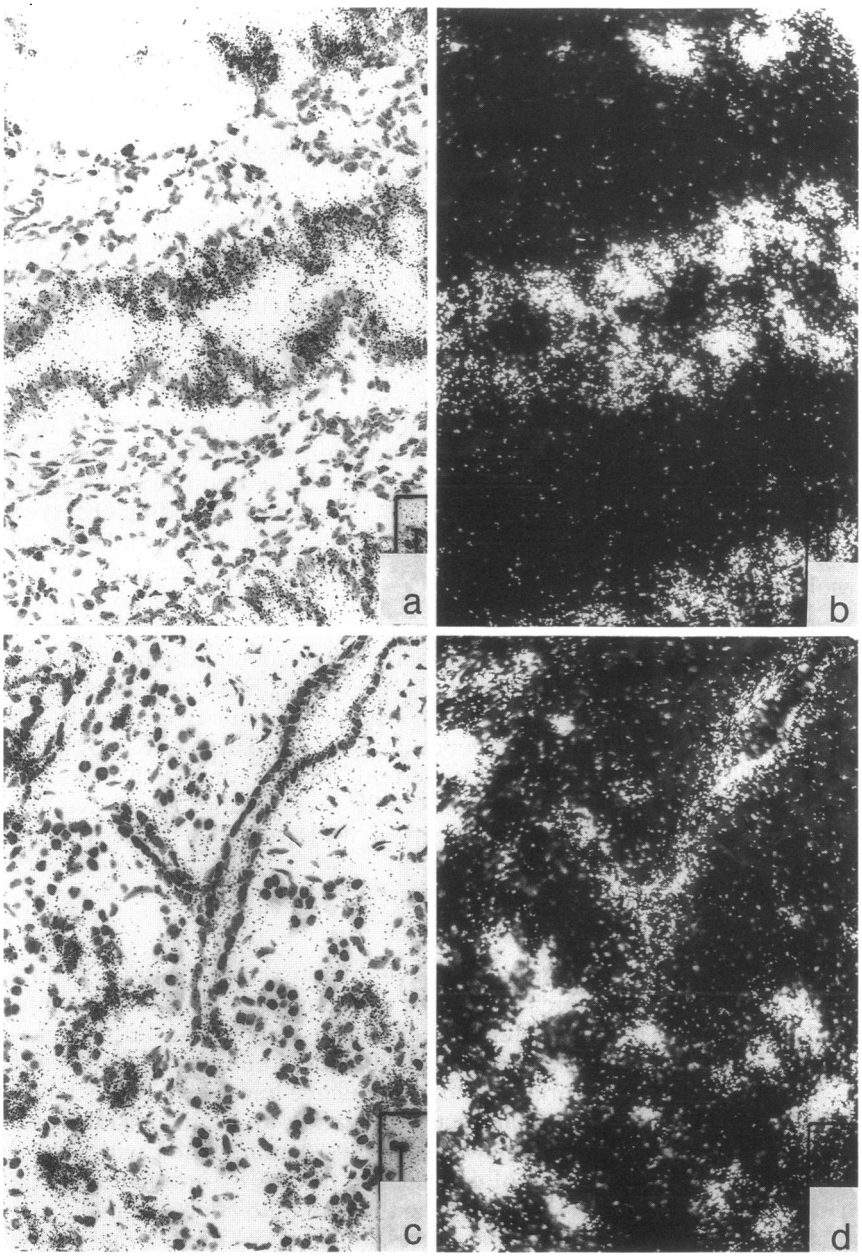


**Figure 4.** In situ hybridization of human tissue sections with antisense osteopontin probes. Magnification is indicated by bars that equal 25 μm. (a and b) Secretory endometrium, osteopontin antisense probe: bright-field (a) and dark-field (b). Note intense labeling of secretory glandular epithelium. (c and d) Pancreas, osteopontin antisense probe: bright-field (c) and dark-field (d). Note labeling of large duct (running from upper right to center) and smaller surrounding ducts. Hybridization performed with control sense probe gave low backgrounds without any epithelial labeling pattern (not shown).

of many epithelia communicating with the external environment are particularly significant and suggest that this protein plays an important role at these surfaces.

The relatively high levels of osteopontin mRNA in gall bladder, relative to bowel mucosa, together with the presence of osteopontin in bile, raise the possibility that osteopontin secreted into bile may contribute much of the osteopontin detected on the mucosal surfaces of the small and large intestines. Moreover, our data suggest that osteopontin at these sites may exist in polymeric forms. Immunoblotting of bile proteins identified not only osteopontin and osteopontin fragments but also high molecular weight complexes. These high molecular weight complexes did not dissociate in the presence of SDS, reducing agent, and heat and thus may have resulted from covalent cross-linking of osteopontin either to itself or to other bile components. Osteopontin has been shown previously to be a substrate for hepatic transglutaminase that catalyzes cross-linking of glutamine and lysine residues (Beninati *et al.*, 1991; Prince *et al.*, 1991).

In the adult human kidney, osteopontin transcripts were found predominantly in the epithelial cells of distal tubules. By immunohistochemistry, osteopontin was

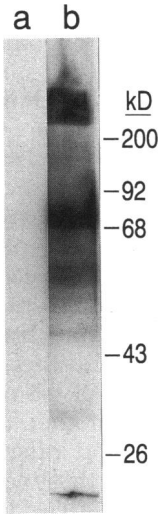


**Figure 5.** Autoradiogram of an immunoblot of human bile proteins stained with osteopontin antibody, and [$^{125}$I]protein A. Bile proteins were fractionated by adsorption to barium citrate, electrophoresed on a 8.5% polyacrylamide gel, transferred to nitrocellulose, and stained with control (a) or osteopontin antiserum (b) (see MATERIALS AND METHODS). Positions of molecular weight markers are indicated: myosin (H-chain), 200 kDa; phosphorylase B, 92 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; $\alpha$-chymotrypsinogen, 26 kDa. The broad band with leading front at 68 kDa corresponds to the mobility of monomeric osteopontin purified from human milk (Senger *et al.*, 1989b). Exposure time: 36 h.

localized primarily to the luminal surfaces of distal tubules, collecting ducts, and to the surfaces of transitional epithelium of the renal pelvis and bladder. A slightly different distribution has been reported in rodent kidney. In the mouse kidney, Nomura *et al.* (1988) reported osteopontin transcripts in epithelium of both proximal and distal tubules, and in rats, Ullrich *et al.* (1991) reported osteopontin along the luminal surfaces of both proximal and distal tubules. Although we cannot rule out low level expression of osteopontin by proximal tubules, it was clear that the epithelia of the distal tubules were the major site of synthesis in human kidney. Osteopontin recently has been found in human urine (Shiraga *et al.*, 1992), raising the possibility that some of the protein synthesized is released into the lumen. Thus, osteopontin is present in at least three bodily fluids, including urine, bile (Figure 5), and milk (Senger *et al.*, 1989b). Our immunohistochemical findings also raise the possibility that osteopontin is secreted by epithelial cells into saliva and sweat.

Osteopontin mRNA expression has been found to be elevated in the skin and breast tissue of pregnant and lactating mice, and expression in the skin of nonpregnant mice has been induced by topical administration of estrogen and progesterone (Craig and Denhardt, 1991). Consistent with those observations, we found markedly stronger immunostaining for osteopontin in lactating human breast as compared with nonlactating breast. Also consistent with hormonal induction, hypersecretory endometrial glands associated with pregnancy stained with particular intensity and more strongly than the secretory endometrium in the absence of pregnancy. In this study, we did not examine the effect of pregnancy on osteopontin expression in the skin; however, we did determine with immunostaining that osteopontin is predominantly, and perhaps exclusively, restricted in skin to the sweat glands and the luminal surface of sweat ducts. To our knowledge, the cellular location of osteopontin in rodent skin has not been determined.

Although our study of human tissues revealed that osteopontin is widely expressed by epithelium, we also

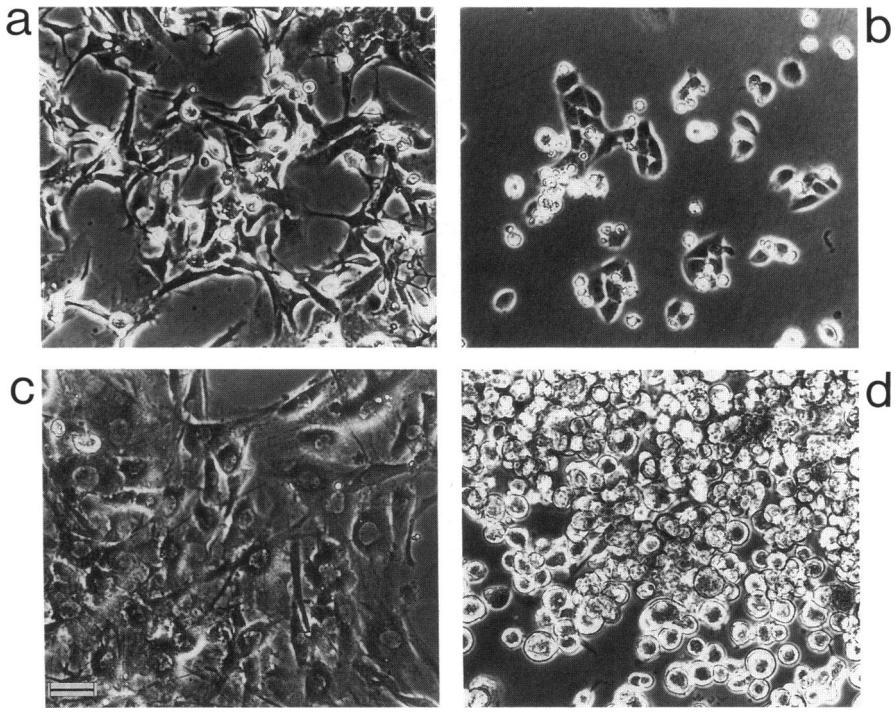


**Figure 6.** Cell attachment and spreading on osteopontin purified from human breast milk. (a–c) Wells were coated first with osteopontin (6 $\mu$g/ml), washed, and then incubated with bovine serum albumin (BSA) at 20 mg/ml. Cells were photographed 3–5 h after plating; cell spreading persisted beyond 24 h. (a) CCD-18 Co, human colon fibroblasts; (b) K16, rat liver epithelial cells; (c) HISM, human intestinal smooth muscle cells; (d) BSA control; HISM cells plated on plastic coated with BSA alone did not attach. Similarly, neither CCD-18 Co cells nor K16 cells attached to BSA wells without osteopontin (not shown). Bar at bottom left of (c) is 12 $\mu$m.

detected osteopontin and its transcript in ganglion cells residing between muscle layers of the intestinal tract. Osteopontin expression by rodent auditory ganglia and nerves of the embryonic ear has been demonstrated previously (Mark *et al.*, 1988; Swanson *et al.*, 1989); hence, osteopontin expression may be associated with ganglion cells in a variety of tissues. In contrast to ganglia, we found that the smooth muscle cells and other interstitial cells of large and small bowel wall were negative for both osteopontin and its transcript. Giachelli *et al.* (1991) recently reported that rat aortic smooth muscle cells in culture express high levels of osteopontin mRNA; in contrast, we found no evidence of osteopontin or osteopontin transcript in association with smooth muscle of the various organs we examined, but we did not investigate aorta.

The finding of a GRGDS cell-binding domain in osteopontin provided the first indication that this protein might play a role in cell adhesion (Oldberg *et al.*, 1986); in particular, it appears likely that osteopontin promotes the anchorage of osteoclasts to bone matrix. Cells attach to and spread on immobilized bone osteopontin (Somerman *et al.*, 1989), and intense osteopontin staining has been found by immunohistochemistry on bone surfaces at sites of osteoclast anchorage (Reinholt *et al.*, 1990). Moreover, soluble osteopontin and an osteopontin peptide, including the RGD domain, inhibited osteoclast binding to bone particles and also inhibited bone resorption in vitro (Miyauchi *et al.*, 1991). Thus, osteopontin associated with bone matrix may promote osteoclast adhesion and resorption of matrix. The role of osteopontin in epithelium is unknown; moreover, there may be structurally different forms of osteopontin with different functions. Multiple forms of osteopontin have been described, including differentially glycosylated species (Singh *et al.*, 1990a), forms with and without phosphorylation (Nemir *et al.*, 1989; Chang and Prince, 1991), with and without sulphation (Kubota *et al.*, 1989; Nagata *et al.*, 1989), as well as naturally occurring proteolytic fragments (Senger *et al.*, 1988; Senger *et al.*, 1989a; Zhang *et al.*, 1990; Ullrich *et al.*, 1991), and, as shown here, polymeric forms. There is also evidence supporting differential splicing of osteopontin transcripts, although there is no evidence, so far, for variability in expression of these spliced transcripts among tissues (Young *et al.*, 1990). In short, the extent to which different forms of osteopontin have different functional activities or restricted tissue distributions remains to be determined. However, it is clear that osteopontin derived from both epithelium and bone has a functional RGD cell-binding domain. As reported here, osteopontin expressed by breast epithelium and purified from human milk promoted attachment and spreading of a variety of cell types, including epithelial cells, and soluble GRGDS peptide blocked this process. Thus, osteopontin isolated from either bone (Somerman *et al.*, 1989) or from breast milk behaved in similar fashion as adhesive proteins. Although there are alternative possibilities, a likely hypothesis is that osteopontin binds luminal epithelial cell surface integrins via its RGD domain.

The finding that osteopontin is widely distributed on surfaces of specific populations of epithelial cells in the gastrointestinal tract, bile ducts, pancreatic ducts, urinary and reproductive tracts, breast, salivary glands and ducts, sweat glands and ducts, and bronchi suggests that this protein may have a protective role in interactions between these epithelial surfaces and the external environment. If this were the case, the osteopontin present in breast milk might have a similar role in coating and thereby protecting the gastrointestinal epithelium of the nursing baby and the breast epithelium of the mother. Some bacterial strains have been reported to bind integrins on epithelial-like cells and thereby invade these cells in culture (Isberg and Leong, 1990; Falkow, 1991; Isberg, 1991). Osteopontin might serve to prevent bacterial attachment through competition for these same receptors. Other observations also have suggested that osteopontin may play a role in host defense. For example, the mouse osteopontin gene has been localized to chromosome 5, in the region of *Ric*, a gene that confers *Rickettsia* resistance (Fet *et al.*, 1989), and the possibility remains that *Ric* is an allele of the osteopontin gene. Moreover, elevated concentrations of osteopontin have been observed in the blood of patients with gram-negative bacterial sepsis (Senger *et al.*, 1988), and interleukin-1 has been reported to increase osteopontin expression in vitro (Jin *et al.*, 1990). Although we did not examine inflammatory conditions in this study, others have reported that activation of both T-lymphocytes and macrophage-like cells leads to marked increases in osteopontin mRNA levels (Patarca *et al.*, 1989; Miyazaki *et al.*, 1990), and intradermal administration of osteopontin is reported to elicit a cellular infiltrate consisting primarily of macrophages (Singh *et al.*, 1990b).

In summary, this report provides the first comprehensive description of osteopontin expression and protein distribution in normal adult human tissues. Immunohistochemistry, in situ hybridization, and Northern analyses revealed a previously unsuspected widespread epithelial distribution of osteopontin and osteopontin mRNA. Particularly striking was the concentration of a prominent band of osteopontin staining at many epithelial surfaces communicating with the external environment. We postulate that at these sites, osteopontin binds to integrin receptors located on the luminal epithelial surface by way of its functional RGD cell-binding domain and there may exert a protective function. Ongoing studies seek to identify osteopontin receptors on epithelial cells and to elucidate osteopontin function on these epithelial surfaces in greater detail.

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